Journal Pre-proof

Characterization of the bacteriocins and the PrtR regulator in a plant-associated *Pseudomonas* strain

Maricruz Fernandez, Agustina Godino, Analía Príncipe, Viviana Lopéz Ramírez, José Miguel Quesada, Virginia Rigo, Manuel Espinosa-Urgel, Gustavo M. Morales, Sonia Fischer

PII: S0168-1656(19)30913-7

DOI: https://doi.org/10.1016/j.jbiotec.2019.11.003

Reference: BIOTEC 8542

To appear in: *Journal of Biotechnology*

Received Date: 4 April 2019

Revised Date: 16 September 2019

Accepted Date: 3 November 2019

Please cite this article as: Fernandez M, Godino A, Príncipe A, Lopéz Ramírez V, Quesada JM, Rigo V, Espinosa-Urgel M, Morales GM, Fischer S, Characterization of the bacteriocins and the PrtR regulator in a plant-associated *Pseudomonas* strain, *Journal of Biotechnology* (2019), doi: https://doi.org/10.1016/j.jbiotec.2019.11.003

Characterization of the bacteriocins and the PrtR regulator in a plant-associated *Pseudomonas* strain

Maricruz Fernandez[a,*], Agustina Godino[b,*], Analía Príncipe[b], Viviana Lopéz Ramírez[a], José Miguel Quesada[c], Virginia Rigo[b], Manuel Espinosa-Urgel[c], Gustavo M. Morales[d], and Sonia Fischer[a, **]

[a]*Departamento de Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto-INIAB CONICET, Agencia Postal No. 3, X580BYA Río Cuarto, Córdoba, Argentina*

[b]*Departamento de Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Agencia Postal No. 3, X580BYA Río Cuarto, Córdoba, Argentina*

[c]*Departamento de Protección Ambiental, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Granada, Spain*

[d]*Departamento de Química, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto-IITEMA CONICET, Agencia Postal No. 3, X580BYA Río Cuarto, Córdoba, Argentina*

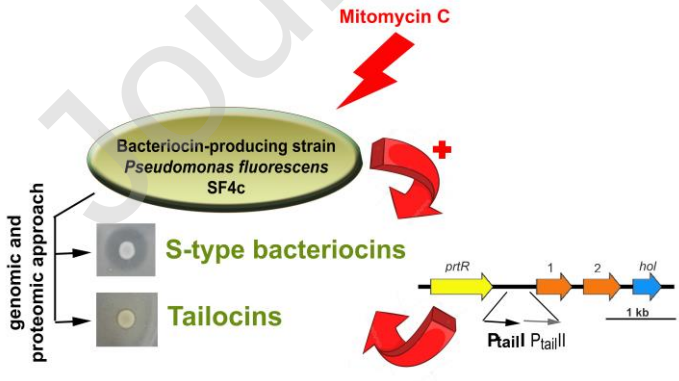
[*]´Fernandez and Godino should be considered joint first authors´

[**]Corresponding author:

Telephone: +54 358 4676438

Email: sfischer@exa.unrc.edu.ar (S. Fischer)

Graphical abstract



Highlights

- *Pseudomonas fluorescens* SF4c produces more than one functional bacteriocin.
- The tailocin promoter is activated during the SOS response.
- PrtR regulates bacteriocin expression positively in *P. fluorescens* SF4c.

Abstract

The emergence of antibiotic resistant bacterial strains demands the development *of* new antimicrobial agents. In the last decades, bacteriocins have gained significant interest due to their potential application as biopreservatives in the food industry and as therapeutic agents in medicine. Recent studies project the use of these antimicrobials in agriculture as biocontrol agents. The characterization of bacteriocins and their genetic regulation, however, have been scarcely studied in plant-associated bacteria.

In this report, an *in-silico* and proteomic analysis was performed to identify the bacteriocins produced by *Pseudomonas fluorescens* SF4c. More than one functional bacteriocin was detected in this strain (S-type bacteriocins and phage-tail-like bacteriocins [tailocins]). It is known that the regulator PrtR represses bacteriocin production in *P. aeruginosa* under normal condition. However, the mechanism for tailocin regulation remains unknown in plant-associated pseudomonads. In this work, an orthologue of the *prtR* of *P. aeruginosa* was identified in the SF4c-tailocin cluster and a *prtR* null mutant constructed. The expression and production of tailocins was abolished in this mutant; thus evidencing that, unlike *P. aeruginosa*, PrtR is a positive regulator of tailocins expression in *P. fluorescens*.

Key words: bacteriocin, pyocin, tailocin, regulation, promoter, *Pseudomonas fluorescens*

1. Introduction

Bacteriocins are proteinaceous toxins that enable the producing organisms to defend their habitat against invaders, limit the advance of neighboring cells, or invade an established bacterial community (Majeed et al., 2013). Within the *Pseudomonas* genus, the best known bacteriocins are the pyocins produced by *P. aeruginosa*. R- and F-type pyocins resemble bacteriophage tails: the R-type pyocins are nonflexible and contractile, whereas those of the F-type are flexible but noncontractile. The S-type pyocins are rather small proteins similar to colicin (bacteriocin produced by *Escherichia coli*), water-soluble and protease- and heat-sensitive. These bacteriocins are secreted as binary-protein complexes

consisting of a protein with killing activity and a protein conferring immunity that remains tightly bound to the cytotoxic domain of the former (Ghequire and De Mot, 2014; Michel-Briand and Baysse, 2002; Scholl, 2017). In most S-type bacteriocins, the amino-terminal domain of the large protein has a receptor-binding function and the carboxy-terminal domain the killing activity, while the central domain mediates translocation to susceptible cells (Ghequire and De Mot, 2014; Parret and De Mot, 2002).

The expression of R-, F-, and S-type-pyocin genes is controlled by the PrtN activator that binds to the P boxes of pyocin promoters. Under normal conditions, the expression of *prtN* is repressed by PrtR. Upon exposure to stress conditions, such as DNA damage by chemicals or ultraviolet irradiation, an activated RecA triggers autoproteolytic cleavage of PrtR, which abrogates *prtN* repression and leads to pyocin production (Matsui et al., 1993; Penterman et al., 2014). A lysis cassette, that encodes a holin and endolysin, mediates the extracellular release of R-pyocin particles in *P. aeruginosa* (Michel-Briand and Baysse, 2002).

In the last decade, *in-silico* analyses have also revealed that the genes encoding pyocin-like bacteriocins are widely distributed in plant-associated pseudomonads (Dingemans et al., 2016; Fischer et al., 2012; Ghequire et al., 2015; Ghequire and De Mot, 2014; Godino et al., 2016; Hockett et al., 2015; Loper et al., 2012; Mavrodi et al., 2009; Parret and De Mot, 2002). Only a few of these bacteriocins, however, have been described in detail. We have characterized a pyocin S-like bacteriocin synthesized by *Pseudomonas fluorescens* SF39a and a tailocin (a phage-tail-like bacteriocin) produced by *P. fluorescens* SF4c, two isolates from wheat rhizospheres that promote growth in plants. These bacteriocins have antimicrobial activity against several phytopathogenic strains of the genus *Xanthomonas* and *Pseudomonas* (Fernandez et al., 2017; Fischer et al., 2012; Godino et al., 2016; Príncipe et al., 2018). Recently, Dorosky *et al.* (2017) demonstrated that *Pseudomonas chlororaphis* strain 30–84 can produce two different R-tailocins with differing killing spectra, both of which contribute to this strain's competition with other rhizosphere-associated bacteria.

In addition to the pyocins, other types of bacteriocins have been discovered in the genus *Pseudomonas*, such as the lectin-like bacteriocin in *Pseudomonas putida* BW11M1, *Pseudomonas protegens* Pf-5 and *Pseudomonas syringae* (Ghequire et al., 2012; Ghequire and De Mot, 2014; Parret et al., 2005, 2003), and colicin M-like bacteriocins in *P. aeruginosa*, *P. syringae* and *P. fluorescens* (Barreteau et al., 2009).

The diversity of bacteriocins in plant-associated *Pseudomonas* strains suggests that these compounds may play a relevant role in the competitiveness of the bacteria (Loper et al., 2012). Moreover, as a significant part of that scenario, the bacteriocins could be employed in the biocontrol of phytopathogenic bacteria (Grinter et al., 2012; Príncipe et al., 2018).

In the present work, an *in-silico* analysis was performed to predict bacteriocin genes in the genome of *P. fluorescens* SF4c. In addition, a proteomic assay was carried out to identify the putative bacteriocin proteins. In an attempt to study the regulation of bacteriocin in *P. fluorescens*, we constructed a *prtR* null mutant and found production to be abolished in that mutant. We additionally demonstrate that the SF4c tailocin promoter is activated during SOS response.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

Pseudomonas and *Xanthomonas* strains were grown in Luria-Bertani medium (LB) at 30 °C. *Escherichia coli* was grown in LB medium at 37 °C. Table 1 lists the bacterial strains used in this study and itemizes the abbreviations used for the different antibiotic-resistance markers.

2.2. Prediction of bacteriocin genes

BAGEL 3 and 4 (**BA**cteriocin **GE**nome mining too**L**; (van Heel et al., 2013) were used to identify *in-silico* genes encoding S-type pyocins, M-type pyocin, and the plant lectin-like bacteriocin (*llpA*) in the genome of *P. fluorescens* SF4c. An additional study was done to seek *llpA* genes, encoding proteins consisting of a tandem of monocot mannose-binding-lectin domains, in the predicted proteome of strain SF4c using the BlastP tool with the LlpA sequence of *P. putida* BW11M1 (GenBank: AAM95702.1) (Ghequire et al., 2012). BAGEL does not contain criteria for bacteriophage-derived bacteriocins, such as the R- and F-type pyocins produced by *P. aeruginosa* (Hockett et al., 2015). Therefore, structural genes encoding tailocins were searched for in the region between the *mutS* and *cinA-recA-recX* loci. The predicted S-type bacteriocin and tailocin genes were further analyzed by the bioinformatics tools open-reading-frame (ORF) finder, BlastP, Pfam, InterPro, and various resources of the ExPASy portal. Cytotoxic domains of predicted S-type bacteriocins were phylogenetically analyzed by the neighbor-joining method through the

use of the MEGA7 software (Kumar et al., 2016). Phylogenetic analyses included the cytotoxic-domain sequences of characterized S-type pyocins of *P. aeruginosa*.

2.3. *Bacteriocin assay*

Ten µl of a stationary-phase culture of *P. fluorescens* SF4c was spotted onto solid LB medium and incubated overnight at 30 ºC. To prevent further cell growth, the plates were then exposed to chloroform vapor (20 min) and overlaid with 3 ml of soft agar (0.7% [w/v]) seeded with 50 µl of a bacterial suspension of the indicator strain. The plates were incubated at 30 ºC and bacteriocin production determined by examining growth inhibition around the colonies (Parret et al., 2003) of the strain *P. fluorescens* SF4c. Strains sensitive to the bacteriocins were used as indicators.

2.4. Bacteriocin-extract preparation

Pseudomonas fluorescens SF4c was grown in two flasks containing 100 mL of LB medium. At an OD_{600} of 0.3, one of the flasks was induced with mitomycin C (final concentration of 3 µg mL^{-1}) and was incubated overnight; the other (with no mitomycin) was incubated for 72 h. Thereafter, the cells and debris were removed by centrifugation at 17,000 *g* for 1 h at 4 °C. The resulting supernatants were precipitated with ammonium sulfate (60%, [w/v]) for 1 h and centrifuged at 17,000 *g* for 1 h at 4 °C. The pellet was resuspended in 5 mL of TN50 buffer [50 mM NaCl, 10 mM Tris/HCl (pH 7.5)] (Fischer et al., 2012; Hockett and Baltrus, 2017; Scholl and Martin, 2008). The antimicrobial activity of the samples was assayed by the spot-test method (Parret et al., 2003) against the sensitive strains *X. vesicatoria* Xcv Bv5-4a, *P. fluorescens* SF39a, or *P. fluorescens* CTR212. The bacteriocin titer was expressed in arbitrary units per ml (AU mL^{-1}), corresponding to the reciprocal of the highest dilution that resulted in a clear inhibition of the sensitive strain (Williams et al., 2008).

2.5. Thermal stability of bacteriocins

Aliquots of bacteriocin extracts were heated at 30 °C or 37 °C for 1 or 3 h, and at 45 °C, 50 °C, 55 °C, and 75 °C for 15 min (Godino et al., 2016). After treatment, the samples were tested for antimicrobial activity against *P. fluorescens* SF39a and *X. vesicatoria* Xcv Bv5-4a as described above.

2.6. Nanoscale liquid-chromatography-tandem-mass-spectrometry (nanoLC-MS/MS) and data analysis

Bacteriocin extracts (from cells induced with mitomycin C and uninduced) were purified as described in section 2.4, lyophilized and subjected to protein reduction (10 mM DTT, 45 min at 56 °C) and alkylation (55 mM iodoacetamide, 45 min in the dark). Next, the samples were digested with trypsin and desalted with Zip-Tip™ C18. Each sample was lyophilized by Speed Vac, resuspended in 10 µl of 0.1% (v/v) formic acid and analyzed by nanoLC-MS/MS. Mass-spectrometry analysis was performed on a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Scientific) coupled to an EASY-nLC™ 1000 Liquid Chromatograph (Thermo Scientific). The ionization was performed by electrospray (Thermo Scientific EASY-Spray™). Data analysis was performed by means of the Proteome Discoverer 1.4 software searching against the proteome predicted from SF4c genome. The search criteria used were: enzyme, trypsin; error maximum, two missed cleavages; MS tolerance, 10 ppm; MS/MS tolerance, 0.05 Da; carbamidomethyl (C), as static modifications; oxidation (M), as dynamic modification; confidence peptides, high (Mass-Spectrometry Service of the Center of Chemical and Biological Studies by Mass Spectrometry [CEQUIBIEM], University of Buenos Aires [UBA]).

The relative abundances of the proteins in mitomycin-C-induced *vs*. uninduced samples was estimated from the counts of peptide spectrum matches (PSMs) attributable to each protein. First, the PSMs were normalized on the basis of the five abundant proteins that remained constant under both experimental conditions. Next, the PSMs of each bacteriocin obtained in the mitomycin-C-induced sample were normalized to the PSMs of the same bacteriocin in the uninduced sample. Only the bacteriocins that were found in both samples and with high-confidence peptides were selected for the analysis.

2.7. Reverse-transcription polymerase chain reaction (RT-PCR) analysis

Total RNA of the strain SF4c was isolated with Tri-Reagent™ (Ambion) and pretreated with the Ambion Turbo™ DNase (Life Technologies) plus 40 U of the RNAase inhibitor RNaseOUT™ (Invitrogen).

The RT-PCR was performed with the Titan OneTube RT-PCR™ kit (Roche Laboratories) according to the recommendations of manufacturer. The primers were designed from the sequences located between or within the locus tag QS95_21665, locus tag QS95_21670, and the locus tag QS95_21675 (*hol gene*). Table S1 lists the sequences

of the primers. Control reactions consisted of: (i) Samples with all the RT-PCR reagents, except the reverse transcriptase (but with Taq polymerase) to check the absence of contaminating genomic DNA in the samples (negative control) (ii) Samples without target RNA and reverse transcriptase, but with DNA and Taq polymerase to ensure proper amplification (positive control) iii) Samples with all the RT-PCR reagents, except RNA (negative control).

2.8. Bioinformatics analysis and tailocin-promoter activity

The BPROM bacterial-promoter-prediction program (http://linux1.softberry.com/berry.phtml) was used to identify possible promoters in the tailocin operon. The putative promoter sequences were amplified from the genomic DNA of the strain SF4c. The first a (P_{tailI}) nd second (P_{tailII}) predicted promoters were amplified through the use of primers p_{tail}I-up and p_{tail}I-down and primers p_{tail}II-up and p_{tail}II-down, respectively. In addition, a region that included both promoters (P_{tail}) was obtained by PCR with primers p_{tail}I-up and p_{tail}II-down. The amplifications were carried out in 25 µl reaction mixtures contained 2.5 µl of 10x PCR buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl), 2.5 mM $MgCl_2$, 0.2 mM of each of the four dNTPs, 0.4 µM of each primers, 0.04 U Taq DNA polymerase (Thermo Fischer Scientific) and 3 µl of DNA. Thermal cycling conditions for amplification of P_{tailI} and P_{tailII} were: one cycle at 95°C for 2 min, then 35 cycles at 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s and a final extension step at 72 °C for 5 min. For amplification of P_{tail}, the thermal cycling conditions were: one cycle at 95 °C for 5 min, then 35 cycles at 95 °C for 40 s, 56 °C for 40 s and 72 °C for 1 m and a final extension step at 72 °C for 10 min. The PCR products obtained were digested with the corresponding enzymes and cloned into pMP220 to obtain pMP220::$P_{tail,}$ pMP220::P_{tailI}, and pMP220::P_{tailII}. In addition, the P_{tail} PCR product was digested with *Eco*RI and *Bam*HI and cloned into pPROBE-TT' containing the *gfp* gene encoding the green-fluorescent protein (GFP) as a marker of putative promoter activities. Recombination in the plasmids was confirmed by PCR. The recombinant plasmids were introduced into the wild-type or mutant strain by electroporation (Choi et al., 2006).

ß-Galactosidase activity was assayed in permeabilized cells by measuring the rate of hydrolysis of *o*-nitrophenyl-ß-D-galactopyranoside (Miller, 1972). Expression of GFP was monitored through the use of a Luminescence Spectrometer at an excitation wavelength of 490 nm and emission wavelength of 510 nm. The data obtained were

expressed as fluorescence arbitrary units and normalized at OD_{600} nm to avoid differences in the growth that might lead to erroneous conclusions.

For both types of measurement, the strains were grown in liquid LB medium at 30 °C with shaking (150 r.p.m.) until an OD_{600} of 0.6. Then, mitomycin C was added and the incubation continued until an OD_{600} of 1.3. For measurement of GFP expression, the bacteria were harvested and washed once with buffered saline. Each assay represented three technical replicates from each of three biological replicates.

2.9. Construction of a prtR mutant and complementation analysis.

A mutant in the *prtR* gene (QS95_RS21660) derivative of *P. fluorescens* SF4c was constructed by replacement of an internal 506 bp fragment of *prtR* with a Km-resistance cassette. For this step, two fragments were PCR-amplified, one with the oligonucleotide primers frag1prtRfw and frag1prtRrv and the other with the oligonucleotides frag2prtRfw and frag2prtRrv (Table S1). The PCR products obtained were digested with *Hind*III/*Bam*HI and *Bam*HI/*Kpn*I, respectively and cloned into pUC18Not (Herrero et al., 1990) to give pPRTR2 and pPRTR21. A *Bam*HI fragment carrying a Km-resistance cassette from plasmid p34S-Km3 (Dennis and Zylstra, 1998) was then cloned into pPRTR21, yielding plasmid pPRTR21Km. This plasmid was digested with *Not*I, and the *prtR::Km* construct was ligated into pKNG101, a suicide vector unable to replicate in *Pseudomonas* spp. that enabled the generation of double recombinations (Kaniga et al., 1991). The resulting plasmid, pKNGPRTR, was introduced into strain SF4c by electroporation. Transformants in which the construct was integrated into the chromosome by a single crossover were selected on LB plates supplemented with Km. The second crossover was selected by plating Km^R clones on medium containing 7% (w/v) sucrose and Km. Moreover, plasmid loss was tested on plates with streptomycin. Km^R Sac^R Sm^S colonies were checked by PCR, Southern hybridization (Sambrook et al., 1989), and nucleotide sequencing to confirm the replacement of *prtR* by *prtR*::Km.

For complementation analysis, the *prtR* gene was introduced as a single copy into the chromosome of mutant *P. fluorescens* SF4c-prtR by means of a mini-Tn7 delivery system. For this step, a fragment containing the coding region of *prtR* and 300 bp upstream from this gene was amplified by PCR from *P. fluorescens* SF4c genomic DNA with the primers prtR1fw and prtR1rv (Table S1). The PCR product was purified, digested with *Kpn*I and *Spe*I, and cloned into the pME3280a vector (Zuber et al., 2003). The construct obtained, pPrtR, and the pUX-BF13 vector (a helper plasmid) were used to transform *P. fluorescens*

SF4c-prtR by electroporation. Plasmid pUX-BF13 carried the genes encoding the transposition proteins necessary for insertion of the Tn7 cassette into the genomic target site (Bao et al., 1991). Insertion of the mini-Tn7 transposon into the specific attTn7 site located downstream of the *glmS* gene, was confirmed by a PCR using primers prtrc (Table S1), which to the inserted DNA, and glms (Table S1), annealing to the 3' end of the *glmS* locus (Miller et al., 2000).

The tailocins from wild-type and mutant strains induced with mitomycin C and uninduced were purified as described in section 2.4, but an additional centrifugation at 58,000 *g* for 1 h at 4 °C was performed to obtain only the tailocin particles. Finally, the pellets were resuspended in 1.5 mL of TN50 buffer (Fischer et al., 2012; Scholl and Martin, 2008). The antimicrobial activity of the samples was assayed by the spot-test method. In addition, the expression of tailocin was studied in this mutant. For this determination, the promotor activity of the mutant and complemented mutant carrying the plasmid pPROBE::Ptail (Table 1) was measured and compared with the GFP expression of the wild-type strain (carrying pPROBE::Ptail). Bacteria carrying the empty plasmid pPROBE-TT' were used as a negative control. Each determination represented three technical replicates from each of three biological replicates.

2.10. Statistical analysis

Statistical analyses were performed by the appropriate parametric procedures with the R software (http://www.r- project.org/). The data were analyzed by the one-way analysis of variance (ANOVA) and comparisons of the means among the treatments made by means of Tukey's *post-hoc* test ($P \leq 0.05$).

3. Results

3.1. Identification of genes involved in bacteriocin production

An *in-silico* genome-mining study was performed to predict bacteriocin genes in the draft genome of strain SF4c through the use of BAGEL, a web-based bacteriocin genome-mining tool that involves an identification approach combining direct mining for the bacteriocin genes and indirect mining via context loci (van Heel *et al.*, 2013). Two genes encoding bacteriocins similar to the S-type pyocins from *P. aeruginosa* were identified in contig 12 (locus tag QS95_RS15610) and contig 5 (locus tag QS95_RS05790) (Fig. 1, Panel A and Table S2). These genes were named *pyoSF4c1* and *pyoSF4c2*, respectively.

The deduced pyocin S-like protein (PyoSF4c1) from locus tag QS95_RS15610 (contig 12) had 692 amino acids, a molecular mass of 75,246.36 Da, and it presented similarity with S-type bacteriocins from *P. putida* NFIX47 (99% similarity), *P. fluorescens* PF_150 (89% similarity), *P. fluorescens* Pf0-1 (54% similarity), pyocin S3 from *P. aeruginosa* P12 (50% similarity), among others. The carboxy-terminal stretch (residues 535 through 692) of PyoSF4c1 was not very similar to any of the characterized S-type pyocins of *P. aeruginosa*, sharing only 23.1% similarity with pyocin S3 cytotoxic domain with DNase activity. This is in agreement with Ghequire and De Mot (2014), who demonstrated that the pyocin S3-like cytotoxic domains, derived from predicted bacteriocin genes, present an extensive diversity in the sequence. In the central region of PyoSF4c1 (residues 401 to 534), there was a Pyocin_S Pfam domain (PF06958) that was present in S-pyocins that had intracytoplasmic targets (such as pyocins with DNase activity) and constituted the translocation domain (Ghequire and De Mot, 2014). The pyocin S-like protein (PyoSF4c2) predicted from locus tag QS95_RS05790 (contig 5) had 214 amino acids and a molecular mass of 23,573.69 Da. This protein showed similarity with S-type bacteriocins from *P. putida* NFIX47 (99% similarity), *P. fluorescens* Pf0-1 (86% similarity), pyocin S2 from *P. aeruginosa* PAO1 (61% similarity), among other. Protein PyoSF4c2 possessed a colicin/pyocin, DNase domain (IPR037146) at the C-terminal (residues 84 through 212). That cytotoxic domain contained the HNH-endonuclease motif (cd00085), which sequence constituted the core of the catalytic site of the endonuclease and was present in most pyocins and colicins with DNase activity (Ghequire and De Mot, 2014; Parret and De Mot, 2002). Moreover, a truncated Pyocin_S translocation domain (PF06958) was found (residues 5 through 79).

The cytotoxic domain of the putative PyoSF4c2 closely resembled (55.8% similarity) the equivalent region of the pyocin AP41 of *P. aeruginosa* (Fig. 1, Panel B). Like this putative bacteriocin, DNase pyocins of *P. aeruginosa* as AP41 S1 and S2 contained the HNH-endonuclease motif and were part of the HNH-DNase-pyocin family (Ghequire and De Mot, 2014; Parret and De Mot, 2002). In contrast, the cytotoxic domain of the putative PyoSF4c1resembled the cytotoxic domain of the DNase pyocin S3 (23.1% similarity) (Fig. 1, Panel B), which sequence did not contain the HNH-motif and lacked homology to the cytotoxic region of members of the HNH-DNase-pyocin family (Parret and De Mot, 2002; Ghequire and De Mot, 2014).

Putative genes involved in immune functions (*immSF4c1* and *immSF4c2*) were also detected in the SF4c genome downstream from *pyoSF4c1* and *pyoSF4c2*, respectively

(Fig. 1, Panel A and Table S2). The predicted ImmSF4c2 protein, with 83 amino acids and a molecular mass of 9,307.45 Da, showed similarity to the bacteriocin immunity proteins from *P. putida* NFIX47 (98% of similarity), *P. fluorescens* Pf0-1 (70% of similarity), *P. aeruginosa* PAO1 (65% of similarity), among other. A colicin/pyocin immunity protein domain (PF01320) was identified in ImmSF4c2 protein (residues 2 through 82). The predicted ImmSF4c1 protein had 86 amino acids and a molecular mass of 9,761.34 Da and showed similarity to the bacteriocin immunity proteins from *P. putida* NFIX47 (98% of similarity), *P. fluorescens* CH267 (96% of similarity), among other. No domain was identified in ImmSF4c1 protein because the Pfam domain PF01320 had been predicted in only the immunity protein of HNH-DNase pyocins (Ghequire and De Mot, 2014).

By contrast, genes encoding M-type pyocins and plant lectin-like bacteriocins were not detected in the draft genome of strain SF4c.

We had previously identified genes encoding a lytic system, holin (*hol*) and endolysin (*lys*) as well as a putative regulatory protein of the cro/cI family (*prtR*) belonging to tailocin cluster in *P. fluorescens* SF4c. Moreover, the conserved genes *mutS* and *recA-recX* was also detected (Fischer et al., 2012; Ly et al., 2015). In *P. fluorescens*, the tailocin genes are located between the conserved *mutS* and *cinA-recA-recX* loci (Fischer et al., 2012; Ghequire and De Mot, 2014; Mavrodi et al., 2009). Therefore, an *in-silico* analysis was performed to find all the genes of the SF4c tailocin cluster located between *mutS* (QS95_RS21655) and the *recX* genes (QS95_RS21890) in the bacterial chromosome. The tailocin cluster was approximately 39.3 kb in length and consisted of 49 putative ORFs (contig 18) (Fig. 2, Panel A and Table S2). In addition to regulatory and lytic genes, the structural genes of the R-type tailocin (from QS95_RS21685 to QS95_RS21755) and F-type tailocin (from QS95_RS21760 to QS95_RS21860) were located.

3.2. Antimicrobial activity and protein identification by mass spectrometry

The antibacterial activity of *P. fluorescens* SF4c was tested against reference strains of the genus *Pseudomonas* and *Xanthomonas* (Fig. 1, Panel C). Strain SF4c produced two types of inhibition halos. In some instances, for example against *P. fluorescens* SF39a, a small inhibition halo characteristic of a high-molecular-mass bacteriocin, such as tailocins, was observed. In others, for example against *X. vesicatoria* Xcv Bv5-4a, a large inhibition zone surrounding the colony of strain SF4c was present, possibly resulting from the action of low-molecular-mass bacteriocins (like the S-like bacteriocins), which are small and

therefore diffuse farther from the colony. In the last example, a halo of smaller diameter could be masked by a larger one.

In addition, the sensitivity of the bacteriocins to heat treatment was analyzed (Fig. 1, Panel D). After incubation at 55 °C for 15 min, the bacteriocin titer against *P. fluorescens* SF39a was maintained at 10^{-3} AU mL^{-1}, but the activity was lost when the bacteriocin was exposed to 75 °C for 15 min. In contrast, the bacteriocin titer against *X. vesicatoria* Xcv Bv5-4a decreased at temperatures above 50 °C. These results suggest that *P. fluorescens* SF4c produces two classes of bacteriocins: a heat-sensitive one of low-molecular-mass (like the S-pyocins) and a heat-resistant form of high-molecular-mass (similar to the tailocins).

To confirm the production of S-type bacteriocins and tailocins from *P. fluorescens* SF4c, a proteomic study was performed. To that end, samples obtained of cultures of strain SF4c (both induced with mitomycin C and uninduced) were analyzed by nanoLC-MS/MS. All together, we identified 32 proteins belonging to the tailocin cluster through the occurrence of high-confidence peptides in at least 1 out 2 sets of nanoLC-MS/MS data, including the structural proteins of the R- and F-tailocins along with the proteins of lytic system holin and endolysin. Within the structural proteins, those of the baseplate, baseplate assembly, phage tail, phage-tail assembly, phage-tail tape measurement, tail sheath, and others were found (Fig. 2, Panel B and Table S2). The same twenty-four structural proteins were found in both samples (induced with mitomycin and uninduced), six of which were identified as structural proteins of R-type tailocins (Fig. 2, Panel C), sixteen as structural proteins of F-type tailocins, and two as other bacteriophage proteins. Moreover, five R- tailocin proteins (a baseplate assembly protein, a phage baseplate protein and three tail proteins) and one F-tailocin protein (a phage tail assembly protein) were only found in the induced sample. The S-type bacteriocins with their cognate immunity proteins were detected through the occurrence of medium-confidence peptides, possibly because of the low proportion of those proteins in the samples.

The relative abundance of bacteriocin proteins that were found in both samples was compared. In cultures induced with mitomycin C, the abundance of all the proteins was from 2.7 to 18.6 times higher than in the uninduced samples, depending on the protein in question (Fig. 2, Panel C). These relative abundance data correlated with the bacteriocin titer obtained in those samples: for example, the antimicrobial activity against strain *X vesicatoria* Xcv Bv5-4a was 10^4 AU mL^{-1} in mitomycin-C–induced cultures and 10^2 AU mL^{-1} in uninduced cultures (Fig. 2, Panel D).

Taken together, these results indicate the production of more than one functional bacteriocin by *P. fluorescens* SF4c. These multiple antimicrobials would increase the killing spectrum of the producing strain, thereby enhancing the strain's competitiveness.

3.3. *Detection of the first gene of the SF4c tailocins*

In *P. aeruginosa*, the first gene of the phage-like pyocin cluster is *hol*, the locus encoding a holin protein. The genes that control the expression of this bacteriocin, *prtR* and *prtN*, are located upstream of *hol* and are transcribed in the opposite direction. This region is not conserved between the tailocins of *P. aeruginosa* and plant-associated pseudomonads, and nothing is known about the regulation and the promoters of tailocin genes in the later. *In-silico* studies of the tailocins from plant-associated pseudomonads revealed that no gene homologous to *prtN* is found in the tailocin cluster and that the *prtR* gene (QS95_RS21660) is oriented in same direction as the other tailocin genes (Fischer et al., 2012; Mavrodi et al., 2009). The region upstream from *hol* is highly variable in the *P. fluorescens* species, where, one, two, or no genes can be located between *prtR* and *hol*, depending on the strain. In strain *P. fluorescens* SF4c, two genes were located *in silico* that belonged to other bacteriophage genes (*orf* 1 and *orf* 2; Fig. 3, Panel A). Moreover, sequence analysis revealed a *rho*-independent transcription terminator downstream from *prtR*, suggesting that this gene is transcribed independently from the tailocin cluster. No potential transcriptional terminators were detected among the loci *orf 1*, *orf 2*, and *hol*. Thus, we decided to test if the three genes formed a transcriptional unit, in order to then define the potential regulatory region(s) involved in tailocin production control. RT-PCR was performed with primers designed to amplify the 3' end of the upstream gene, the intergenic region and the 5' end of the downstream gene. Amplicons with the expected sizes were obtained from the cDNA, indicating that *orf 1*, *orf 2*, and *hol* were all three cotranscribed (Fig. 3, Panel B) and suggesting that a promoter upstream *orf1*would direct expression of the cluster.

3.4. Analysis of putative promoters of tailocin

BPROM software (http://linux1.softberry.com/berry.phtml) was used to identify the putative promoters upstream from the *orf 1* gene. Two possible promoters were predicted. The first was located from position -396 to -347 bp and the second from position -73 to -24 bp relative to the *orf 1* start codon (Fig. 4, Panel A). Primers were designed to amplify a region including either both the putative promoters together (P_{tail}) or each promoter

separately (P_{tailI} and P_{tailII}) (Table S1). The amplified promoter regions were cloned into the vector pMP220, which harbors a promoterless *lacZ* gene, and the correct construct confirmed by sequencing. The resulting recombinant plasmids—designated pMP220::P_{tail}, pMP220::P_{tailI}, and pMP220::P_{tailII}—were independently introduced into strain SF4c by electroporation. The promoter activity was measured by a ß-galactosidase assay in the presence or absence of mitomycin C (the bacteriocin inducer). A basal level of activity (of *ca*. 200 Miller units) was detected in the uninduced cells (pMP220::P_{tail}). The expression of P_{tail}, however, increased to more than 2,300 Miller units when the cells were induced with mitomycin C, demonstrating that the tailocin promoter is included in this region and is activated during the SOS response (Fig. 4, Panel B). In addition, the activity of each promoter was also measured. The results from the ß-galactosidase–activity assays demonstrated that P_{tailI} was the primary active promoter for the production of tailocins (at 570 Miller units in cultures induced with mitomycin C). The ß-galactosidase–activity for promoter PtailII was not significantly different in cells induced with mytomicin C and uninduced.

3.5. Role of PrtR in the expression of SF4c tailocins

The deduced PrtR protein from *P. fluorescens* SF4c (locus tag QS95_RS21660) has 247 amino acids and a molecular mass of 27,738.30 Da and presents 60% similarity to the protein's orthologs from *P. aeruginosa*. Moreover, PrtR from strain SF4c present two conserved domains, a Cro/C1- type helix-turn-helix (HTH) domain (residues 13 to 68) capable of binding DNA, present in transcriptional regulators and a peptidase-S24 domain (residues 154 to 222). Those domains are also identified in the PrtR protein of other *Pseudomonas* strains (Figure S2).

In addition, features of known repressor proteins involved in SOS regulation of gene expression are conserved in that PrtR of *P. fluorescens* SF4c. (i) The Gly-Asn-Ser-Met sequence starting at residue 157 to 160 is identical to the sequence conserved among bacteriophage and Lex-A repressors and is proposed to be involved in the cleavage of those repressors (Slilaty and Little, 1987). (ii) The presence in tandem of the Ala-Gly sequence. These residues are involved in the cleavage of the LexA protein and phage repressors (Cys-Gly in the bacteriophage-λ 80 cI repressor) by the RecA protein, activated during the SOS response (Horii et al., 1981). In PrtR from *P. fluorescens* SF4c, this sequence differs in one amino acid (Ala-Gly-Ser-Gly) at residue 118.

A *prtR* mutant derivative of *P. fluorescens* SF4c was constructed by replacement of a *prtR* fragment with a Km-resistance cassette as described in Materials and methods. One double-recombinant clone was selected and named *P. fluorescens* SF4c-prtR and the double crossover confirmed by PCR and Southern blotting. Production of tailocins was analyzed in the mutant SF4c-prtR and wild-type strain against several reference strains. Uninduced cultures of *P. fluorescens* SF4c had a tailocin titer of 10^2 AU mL^{-1} against *P. fluorescens* SF39a and *X. vesicatoria* Xcv Bv5–4a or one of 10^1 AU mL^{-1} against *P. fluorescens* CTR212. The antimicrobial activity against all indicator strains was completely lost in the mutant SF4c-prtR. When the cultures were induced with mitomycin C, the tailocin titer of the wild-type strain increased to 10^4 AU mL^{-1} for all indicator strains, while the mutant SF4c-prtR remained without manifesting any antimicrobial activity (Table 2).

In order to confirm that the altered phenotype in the mutant SF4c-prtR was due to insertional inactivation of the *prtR* gene, a wild type copy of this gene was inserted into the chromosome of the mutant. For this construction, the full-length *prtR* gene from *P. fluorescens* SF4c was cloned into the pME3280a vector; and, together with a helper plasmid, included to effect a successful insertion, was used to transform mutant SF4c-prtR. Tailocin production was measured in the complemented mutant (strain SF4c-prtR-c). For all the indicator strains tested, the antimicrobial activity was partially restored in the complemented mutant SF4c-prtR-c at 10^1 AU mL^{-1}, relative to the full antimicrobial activity of strain SF4c of 10^4 AU mL^{-1}, in comparison to the mutant strain with no activity at all.

To further elucidate the role of PrtR in the regulation of tailocins, promoter-expression assays were performed (Fig. 5). In these experiments, the activity of the P_{tail} promoter was analyzed after introduction of the recombinant plasmid pPROBE::P_{taiI} into strain SF4c, SF4c-prtR, and the complemented mutant (*P. fluorescens* SF4c-prtR-c). This plasmid contains the same tailocin promoter as pMP220::P_{tail}, but cloned upstream a promoterless *gfp* gene, thus providing a facile way to quantify promoter activity through fluorescence measurements. No activity of the promoter was observed in the mutant SF4c-prtR (pPROBE::P_{tail}), whereas the strain SF4c (pPROBE::P_{tail}) exhibited a significantly strong promoter activity (*ca.* 1,100 GFP arbitrary units/$OD_{600\ nm}$). Therefore, both the regulation of expression and the production of tailocin are affected in the mutant SF4c-prtR. The promoter activity in the complemented mutant (pPROBE::P_{tail}) was restored

partially to *ca*. 455 GFP arbitrary units/$OD_{600\ nm}$, thus confirming that the *prtR* gene was involved in the regulation of tailocin synthesis.

4. Discussion

In the present report, genes encoding four bacteriocins (two S-type bacteriocins and two phage tail-like tailocins) were identified in the genome of *P. fluorescens* SF4c. The structural genes of R- and F-type tailocins were detected between the conserved region *mutS* and *cinA-recA-recX*. Strain SF4c contains the tailocin cluster in the same genomic position as other plant-associated pseudomonad strains—those being specifically of the species *P. fluorescens, P. putida*, *P. protegens*, and *P. chlororaphis*. In contrast, the tailocins produced by *P. syringae* and *P. aeruginosa* are integrated between the *trpE* and *trpG* genes, even though those tailocins were derived independently (Dorosky et al., 2017; Ghequire et al., 2015; Hockett et al., 2015; Mavrodi et al., 2009). These correlations suggest that the tailocins from pathogenic strains are found in identical genomic positions, and the same occurs among the beneficial strains of *Pseudomonas* genus. Why integration is favored in that genetic region is not even clear.

In *P. aeruginosa*, the first gene in the cluster of R- and F-type pyocins is *hol*. The genes that control the expression of these bacteriocins, *prtR* and *prtN*, are located upstream of *hol* and are transcribed in the opposite direction (Nakayama et al., 2000). A study comparing the tailocin clusters revealed that the region upstream from *hol* is not conserved among plant-associated *Pseudomonas* (Mavrodi et al., 2009). In addition, the promoter of these tailocins remained unknown until the present time. In this report, we have demonstrated that the *orf 1*, *orf 2*, and *hol* loci are cotranscribed into a polycistronic messenger in *P. fluorescens* SF4c. Therefore, the first gene in the SF4c tailocins is *orf1*. The *orf 1* and *orf 2* genes are related to those of bacteriophages, but no name has been assigned as yet. A bioinformatics study predicted two putative promoters upstream from *orf 1* and both were necessary to attain a higher expression. Moreover, the activity of those tailocin promoters increased when the cultures were induced with mitomycin C. Those findings confirmed the previous results that the SOS response was involved in the expression of tailocins in *P. fluorescens* SF4c (Fischer et al., 2012).

The *prtR* gene was identified in the tailocin cluster of *P. fluorescens* SF4c, though no gene homologous to *prtN* was detected (Fischer et al., 2012). Those results agreed with observations in other tailocin clusters in plant-associated *Pseudomonas*

(Dorosky et al., 2017; Ghequire et al., 2015; Mavrodi et al., 2009). The function of the *prtR* gene in bacteriocin regulation has not been studied beyond the *P. aeruginosa* species. In the present report, a detailed study on the function of *prtR* was performed in *P. fluorescens* SF4c. The PrtR of strain SF4c presents a high degree of similarity to its ortholog in *P. aeruginosa* PAO1 in terms of conserved domains and, characteristic sequences of repressors that were involved in the SOS response. In order to analyze the function of PrtR in the regulation of SF4c tailocin, we constructed a *prtR* null-mutant derivative of *P. fluorescens* SF4c. What was especially interesting was that the tailocin production was completely abolished in this mutant. In addition, the tailocin-promoter activity was not detected in the mutant *prtR*$^{-}$, while the wild-type strain displayed a significantly strong promoter activity. These results suggested that, unlike in *P. aeruginosa*, PrtR regulates expression of tailocins in *P. fluorescens* SF4c in a positive fashion. Moreover, an Operator-like sequence (ATAAATGCATTTAC) was predicted upstream of the -35 region of the first tailocin promoter (P_{tailI}). We speculate that this site could be a binding site for PrtR. However, additional studies will be necessary to confirm this.

Depending on the context, a regulator can act as either a repressor or an activator of transcription. For example, the λ phage repressor protein (CI) is the key component of the 'genetic switch' that allows the transition from lysogenic to lytic cycle. CI contains an N-terminal end which contains a HTH motif that binding to the operator sites, and a C-terminal domain that mediates dimerization as well as a dimer–dimer interaction that results in the cooperative binding of a pair of dimers to adjacent operator sites. CI binds to six operator sites located within the left and right operator regions (OR and OL) of the λ chromosome. The affinity of CI for each of the operator varies; the binding of one dimer to the high-affinity site OR1facilitates the binding of a second dimer to the lower affinity site OR2. The repressor dimer pair cooperatively bound at OR1 and OR2 interacts with a similar dimer pair cooperatively bound at OL1 and OL2, and forms a loop. This binding prevents transcription of the viral genes necessary for lytic growth and activates transcription of its own gene (the *cI* gene) to maintain the lysogenic mode. At high CI concentration, two more dimers bind to the weakest operator sites OR3 and OL3; repressing promoter of CI. This autoregulation ensures a narrow range of CI repressor level to be maintained, which is optimum for stable lysogeny. When the lysogenic cell is exposed to a stress that damages the host DNA, bacterial RecA stimulate the self-cleavage of CI, therefore the N-terminal and C-terminal domains are separate and the repressor is

inactivated. This leads to expression of lytic genes (Bell et al., 2000; Dodd et al., 2001; Johnson et al., 1979; Meyer and Ptashne, 1980; Pabo et al., 1979; Ptashne, 2014; Sauer et al., 1982; Stayrook et al., 2008). Another example is LexA protein. During the normal growth of *E. coli*, LexA dimers binds to operator sequences and represses the expression of at least 43 unlinked genes, including the genes encoding for bacteriocins. After DNA damage, RecA is activated and stimulates self-cleaving activity in LexA. Then, LexA dissociates from its DNA targets, allowing transcription of these genes (Butala et al., 2009; Cascales et al., 2007; Courcelle et al., 2001; Fernández De Henestrosa et al., 2000). In contrast, it has been demonstrated that LexA can also act a transcriptional activator in *Rhodobacter sphaeroides* and *Synechocystis* sp. *cyanobacterium* (Gutekunst et al., 2005; Tapias et al., 2002).

In conclusion, this report describes the characterization of novel bacteriocins and documents for the first time, to the best of our knowledge, the function of PrtR in the regulation of tailocin expression in a plant-associated *Pseudomonas* strain. PrtR could well be regulating the production of tailocins in different way among the various *Pseudomonas* species.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Acknowledgements

This research was supported by the Agencia Nacional de Promoción Científica y Tecnológica (PICT 1499/12; PICT 0850/16), Secretaría de Ciencia y Técnica de la Universidad Nacional de Río Cuarto (18C/471), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET; 11220120100352CO). G.M. Morales and S. Fischer are members of the Scientific Researcher Career-CONICET. M. Fernandez and A. Godino are fellows of CONICET. V. Lopéz Ramírez has a doctoral fellowship from ANPCyT. Dr.

Donald F. Haggerty, a retired academic career investigator and native English speaker, edited the final version of the manuscript.

References

Bao, Y., Douglas, P.L., Hanian, F., Gary, P.R., 1991. An improved Tn7-based system for the single-copy insertion of cloned genes into chromosomes of Gram-negative bacteria. Gene 109, 167–168.

Barreteau, H., Bouhss, A., Fourgeaud, M., Mainardi, J., Touze, T., Ge, F., Blanot, D., Arthur, M., Mengin-lecreulx, D., Bacte, E., Curie, M., F-, P., 2009. Human- and plant-pathogenic Pseudomonas species produce bacteriocins exhibiting Colicin M-like hydrolase activity towards peptidoglycan precursors. J. Bacteriol. 191, 3657–3664. https://doi.org/10.1128/JB.01824-08

Bell, C.E., Frescura, P., Hochschild, A., Lewis, M., 2000. Crystal Structure of the λ Repressor C-Terminal Domain Provides a Model for Cooperative Operator Binding. Cell 101, 801–811.

Butala, M., Žgur-Bertok, D., Busby, S.J.W., 2009. The bacterial LexA transcriptional repressor. Cell. Mol. Life Sci. 66, 82–93. https://doi.org/10.1007/s00018-008-8378-6

Cascales, E., Buchanan, S.K., Duche, D., Kleanthous, C., Lloube, R., Postle, K., Riley, M., Slatin, S., 2007. Colicin biology. Microbiol. Mol. Biol. Rev. MMBR 71, 158–229. https://doi.org/10.1128/MMBR.00036-06

Choi, K.H., Kumar, A., Schweizer, H.P., 2006. A 10-min method for preparation of highly electrocompetent Pseudomonas aeruginosa cells: Application for DNA fragment transfer between chromosomes and plasmid transformation. J. Microbiol. Methods 64, 391–397. https://doi.org/10.1016/j.mimet.2005.06.001

Courcelle, J., Khodursky, A., Peter, B., Brown, P.O., Hanawalt, P.C., 2001. Comparative Gene Expression Profiles Following UV Exposure in Wild-Type and SOS-Deficient Escherichia coli. Genetics 158, 41–64.

Dennis, J.J., Zylstra, G.J., 1998. Improved antibiotic-resistance cassettes through restriction site elimination using Pfu DNA polymerase PCR. Biotechniques. 25(5), 772–776. https://doi.org/10.2144/98255bm04

Dingemans, J., Ghequire, M.G.K., Craggs, M., De Mot, R., Cornelis, P., 2016. Identification and functional analysis of a bacteriocin, pyocin S6, with ribonuclease activity from a Pseudomonas aeruginosa cystic fibrosis clinical isolate. Microbiol. Open 5, 413–423. https://doi.org/10.1002/mbo3.339

Dodd, I.B., Perkins, A.J., Tsemitsidis, D., Egan, J.B., 2001. Octamerization of λ CI repressor is needed for effective repression of P RM and efficient switching from lysogeny. GENES Dev. 15, 3013–3022. https://doi.org/10.1101/gad.937301.presses

Dorosky, J.R., Yu, J.M., Pierson III, L.S., Pierson, E.A., 2017. Pseudomonas chlororaphis produces two distinct R-Tailocins that contribute to bacterial competition in biofilms and on roots. Appl. Environ. Microbiol. 83, 1–16. https://doi.org/10.1128/AEM.00706-17

Fernández De Henestrosa, A.R., Ogi, T., Aoyagi, S., Chafin, D., Hayes, J.J., Ohmori, H., Woodgate, R., 2000. Identification of additional genes belonging to the LexA regulon in Escherichia coli. Mol. Microbiol. 35, 1560–1572. https://doi.org/10.1046/j.1365-2958.2000.01826.x

Fernandez, M., Godino, A., Príncipe, A., Morales, G.M., Fischer, S., 2017. Effect of a Pseudomonas fluorescens tailocin against phytopathogenic Xanthomonas observed by atomic force microscopy. J. Biotechnol. 256, 13–20. https://doi.org/10.1016/j.jbiotec.2017.07.002

Fischer, S., Godino, A., Quesada, J.M., Cordero, P., Jofré, E., Mori, G., Espinosa-Urgel, M., 2012. Characterization of a phage-like pyocin from the plant growth-promoting rhizobacterium Pseudomonas fluorescens SF4c. Microbiology 158, 1493–1503. https://doi.org/10.1099/mic.0.056002-0

Ghequire, M.G.K., De Mot, R., 2014. Ribosomally encoded antibacterial proteins and peptides from Pseudomonas. FEMS Microbiol. Rev. 38, 523–568. https://doi.org/10.1111/1574-6976.12079

Ghequire, M.G.K., Dillen, Y., Lambrichts, I., Proost, P., Wattiez, R., De Mot, R., 2015. Different ancestries of R tailocins in rhizospheric Pseudomonas isolates. Genome Biol Evol 7, 2810–2828. https://doi.org/10.1093/gbe/evv184

Ghequire, M.G.K., Li, W., Proost, P., Loris, R., Mot, R. De, 2012. Plant lectin-like antibacterial proteins from phytopathogens Pseudomonas syringae and Xanthomonas citri 4, 373–380. https://doi.org/10.1111/j.1758-2229.2012.00331.x

Godino, A., Príncipe, A., Fischer, S., 2016. A ptsP deficiency in PGPR Pseudomonas fluorescens SF39a affects bacteriocin production and bacterial fitness in the wheat rhizosphere. Res. Microbiol. 167, 178–189. https://doi.org/10.1016/j.resmic.2015.12.003

Grinter, R., Milner, J., Walker, D., 2012. Ferredoxin containing bacteriocins suggest a novel mechanism of iron uptake in Pectobacterium spp. PLoS One 7, e33033. https://doi.org/10.1371/journal.pone.0033033

Gutekunst, K., Phunpruch, S., Schwarz, C., Schuchardt, S., Schulz-friedrich, R., Appel, J., 2005. LexA regulates the bidirectional hydrogenase in the cyanobacterium

Synechocystis sp . PCC 6803 as a transcription activator. Mol. Microbiol. 58, 810–823. https://doi.org/10.1111/j.1365-2958.2005.04867.x

Herrero, M., Lorenzo, V.D.E., Timmis, K.N., 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. J. Bacteriol. 172, 6557–6567.

Hockett, K.L., Baltrus, D.A., 2017. Use of the Soft-agar Overlay Technique to Screen for Bacterially Produced Inhibitory Compounds. J. Vis. Exp. 119, e55064. https://doi.org/10.3791/55064

Hockett, K.L., Renner, T., Baltrus, D.A., 2015. Independent co-option of a tailed bacteriophage into a killing complex in Pseudomonas. MBio 6, e00452-15. https://doi.org/10.1128/mBio.00452-15

Horii, T., Ogawa, T., Nakatani, T., Hase, T., Matsubara, H., Ogawa, H., 1981. Regulation of SOS Functions: Purification of E. coli LexA Protein and Determination of Its Specific Site Cleaved by the PecA Protein. Cell. 27, 512–522.

Johnson, A.D., Meyer, B.J., Ptashne, M., 1979. Interactions between DNA-bound repressors govern regulation by the λ phage repressor. Proc. Natl. Acad. Sci. USA 76, 5061–5065.

Kaniga, K., Delor, I., Cornelis, G.R., 1991. A wide-host-range suicide vector for improving reverse genetics in Gram-negative bacteria: inactivation of the blaA gene of Yersinia enterocolitica. Gene 109, 137–141. https://doi.org/10.1016/0378-1119(91)90599-7

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7 : Molecular Evolutionary Genetics Analysis Version 7 . 0 for Bigger Datasets. Mol. Evol. Genet. Anal. 33, 1870–1874. https://doi.org/10.1093/molbev/msw054

Loper, J.E., Hassan, K.A., Mavrodi, D. V, Ii, E.W.D., Lim, C.K., Shaffer, B.T., Elbourne, L.D.H., Stockwell, V.O., Hartney, S.L., Breakwell, K., Henkels, M.D., Tetu, S.G., Rangel, L.I., Kidarsa, T.A., Wilson, N.L., Van, J.E., Mortel, D., Song, C., Blumhagen, R., Radune, D., Hostetler, J.B., Brinkac, L.M., Durkin, A.S., Kluepfel, D.A., Wechter, W.P., Anderson, A.J., Kim, Y.C., Iii, L.S.P., Pierson, E.A., Lindow, S.E., Kobayashi, D.Y., 2012. Comparative Genomics of Plant-Associated Pseudomonas spp .: Insights into Diversity and Inheritance of Traits Involved in Multitrophic Interactions. PLoS Genet. 8, e1002784. https://doi.org/10.1371/journal.pgen.1002784

Ly, L.K., Underwood, G.E., Mccully, L.M., Bitzer, A.S., Godino, A., Bucci, V., Brigham,

C.J., Príncipe, A., Fischer, S.E., Silby, W., 2015. Draft genome sequences of Pseudomonas fluorescens strains SF39a and SF4c, potential plant growth promotion and biocontrol agents. Genome Announc. 3, e00219-15. https://doi.org/10.1128/genomeA.00219-15.

Majeed, H., Lampert, A., Ghazaryan, L., Gillor, O., 2013. The weak shall inherit: bacteriocin-mediated interactions in bacterial populations. PLoS One 8, e63837. https://doi.org/10.1371/journal.pone.0063837

Matsui, H., Sano, Y., Ishihara, H., Shinomiya, T., 1993. Regulation of pyocin genes in *Pseudomonas aeruginosa* by positive (*prtN*) and negative (*prtR*) regulatory genes. J. Bacteriol. 175, 1257–1263.

Mavrodi, D. V, Loper, J.E., Paulsen, I.T., Thomashow, L.S., 2009. Mobile genetic elements in the genome of the beneficial rhizobacterium Pseudomonas fluorescens Pf-5. BMC Microbiol. 9. https://doi.org/10.1186/1471-2180-9-8

Meyer, B.J., Ptashne, M., 1980. Gene Regulation at the Right Operator (OR) of Bacteriophage λ II. ORl, OR2, and OR3 : Their Roles in Mediating the Effects of Repressor and cro. J. Mol. BioE 139, 163–194.

Michel-Briand, Y., Baysse, C., 2002. The pyocins of *Pseudomonas aeruginosa*. Biochimie 84, 499–510. https://doi.org/10.1016/S0300-9084(02)01422-0

Miller, J.H., 1972. Assay of β-galactosidase activity., in: Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp. 352–355.

Miller, W.G., Leveau, J.H.J., Lindow, S.E., Biology, M., Hall, K., 2000. Improved gfp and inaZ Broad-host-range promoter-probe vectors. Mol. Plant-Microbe Interact. 13, 1243–1250. https://doi.org/10.1094/MPMI.2000.13.11.1243

Nakayama, K., Takashima, K., Shinomiya, T., Kageyama, M., Kanaya, S., Ohnishi, M., Mori, H., Hayashi, T., 2000. The R-type pyocin of Pseudomonas aeruginosa is related to P2 phage, and the F-type is related to lambda phage. Mol. Microbiol. 38, 213–231.

Pabo, C., Sauer, R.T., Sturtevantt, J.M., Ptashne, M., 1979. The λ repressor contains two domains. Proc. Natl. Acad. Sci. USA 76, 1608–1612.

Parret, A.H.A., De Mot, R., 2002. Bacteria killing their own kind: Novel bacteriocins of Pseudomonas and other γ-proteobacteria. Trends Microbiol. 10, 107–112. https://doi.org/10.1016/S0966-842X(02)02307-7

Parret, A.H.A., Schoofs, G., Proost, P., 2003. Plant lectin-like bacteriocin from a

rhizosphere-colonizing pseudomonas isolate. J. Bacteriol. 185, 897–908. https://doi.org/10.1128/JB.185.3.897

Parret, A.H.A., Temmerman, K., De Mot, R., 2005. Novel lectin-like bacteriocins of biocontrol strain Pseudomonas fluorescens Pf-5. Appl. Environ. Microbiol. 71, 5197–5207. https://doi.org/10.1128/AEM.71.9.5197-5207.2005

Penterman, J., Singh, P.K., Walker, G.C., 2014. Biological cost of pyocin production during the SOS response in Pseudomonas aeruginosa. J. Bacteriol. 196, 3351–3359. https://doi.org/10.1128/JB.01889-14

Príncipe, A., Fernandez, M., Torasso, M., Godino, A., Fischer, S., 2018. Effectiveness of tailocins produced by Pseudomonas fluorescens SF4c in controlling the bacterial-spot disease in tomatoes caused by Xanthomonas vesicatoria. Microbiol. Res. 212–213, 94–102. https://doi.org/10.1016/j.micres.2018.05.010

Ptashne, M., 2014. A genetic switch: phage lambda revisited. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sambrook, J., Fritsch, E.F., Maniatis, T.A., 1989. Molecular Cloning: A Laboratory Manual., 2nd ed. ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sauer, R.T., Rosslll, M.J., Ptashnel, M., 1982. Cleavage of the λ and P22 Repressors by recA Protein*. J. Biol. Chem. 257, 4458–4463.

Scholl, D., 2017. Phage Tail–Like Bacteriocins. Annu. Rev. Virol. 4, 453–467. https://doi.org/10.1146/annurev-virology-101416-041632

Scholl, D., Martin, D.W., 2008. Antibacterial efficacy of R-type pyocins towards Pseudomonas aeruginosa in a murine peritonitis model. Antimicrob. Agents Chemother. 52, 1647–1652. https://doi.org/10.1128/AAC.01479-07

Slilaty, S.N., Little, J.W., 1987. Lysine-156 and serine-119 are required for LexA repressor cleavage: a possible mechanism. Proc. Natl. Acad. Sci. U. S. A. 84, 3987–3991. https://doi.org/10.1073/pnas.84.12.3987

Stayrook, S., Jaru-ampornpan, P., Ni, J., Hochschild, A., Lewis, M., 2008. Crystal structure of the λ repressor and a model for pairwise cooperative operator binding. Nature 452, 2–6. https://doi.org/10.1038/nature06831

Tapias, A., Fernández, S., Alonso, J.C., Barbé, J., 2002. Rhodobacter sphaeroides LexA has dual activity: optimising and repressing recA gene transcription. Nucleic Acids Res. 30, 1539–1546.

van Heel, A.J., de Jong, A., Montalban-Lopez, M., Kok, J., Kuipers, O.P., 2013. BAGEL3: automated identification of genes encoding bacteriocins and (non-

)bactericidal posttranslationally modified peptides. Nucleic Acids Res. 41, W448–W453. https://doi.org/10.1093/nar/gkt391

Williams, S.R., Gebhart, D., Martin, D.W., Scholl, D., 2008. Retargeting R-Type pyocins to generate novel bactericidal protein complexes. Appl Env. Microbiol 74, 3868–3876. https://doi.org/10.1128/AEM.00141-08

Zuber, S., Carruthers, F., Keel, C., Mattart, A., Blumer, C., Pessi, G., Gigot-bonnefoy, C., Schnider-keel, U., Heeb, S., Reimmann, C., Haas, D., 2003. GacS sensor domains pertinent to the regulation of exoproduct formation and to the biocontrol potential of Pseudomonas fluorescens CHA0. Am. Phytopathol. Soc. 16, 634–644. https://doi.org/10.1094/MPMI.2003.16.7.634

Figure legends

Fig. 1. Detection of S-type bacteriocins in *P. fluorescens* SF4c.

Panel A. Schematic representation of the genes encoding putative S-type bacteriocins (*pyoSF4c1* and *pyoSF4c2*, pink arrow), and genes encoding immunity proteins (*immSF4c1* and *immSF4c2*, green arrow) in the SF4c genome.

Panel B. Phylogenetic analysis of the cytotoxic domains of PyoSF4c1 and PyoSF4c2 (pink) and the previously characterized S-pyocins of *P. aeruginosa*. Representative pyocins were selected: pyocin AP41 (UniProtKB - Q51502), S1 (UniProtKB - Q06583), and S2 (UniProtKB -Q06584) with DNase (HNH) activity, pyocin M1 (UniProtKB - Q1W548) with lipid-II–degradation activity, pyocin S3 (UniProtKB - Q51549) with DNase (non-HNH) activity, pyocin S4 (UniProtKB - Q9HXE0 (Q9HXE0_PSEAE) with tRNase activity, pyocin S5 (UniProtKB - Q9I4Y4) with pore-forming activity, and pyocin S6 (Dingemans et al., 2016) with rRNase activity.

Panel C. Bacteriocin assay of *P. fluorescens* SF4c against different strains of the genera *Pseudomonas* and *Xanthomonas*. In the table, the qualitative size of the inhibition halo is listed for each of the *Pseudomonas* strains tested. The representative photographs below illustrate the size differences of the two types of growth-inhibition halos. A negative control (resistant strain to bacteriocin) was included.

Panel D. Thermal stability of SF4c bacteriocin assayed against *P. fluorescens* SF39a and *X. vesicatoria* Xcv Bv5-4a. In the figure, the inhibition halos are illustrated at each concentration of bacteriocin in arbitrary units (AU) indicated above the figure from the two strains denoted below after the times of exposure of the proteins to the temperatures listed on the left. The symbol (*) indicates the highest dilution that resulted in a clear inhibition of the sensitive strain. UD: undiluted.

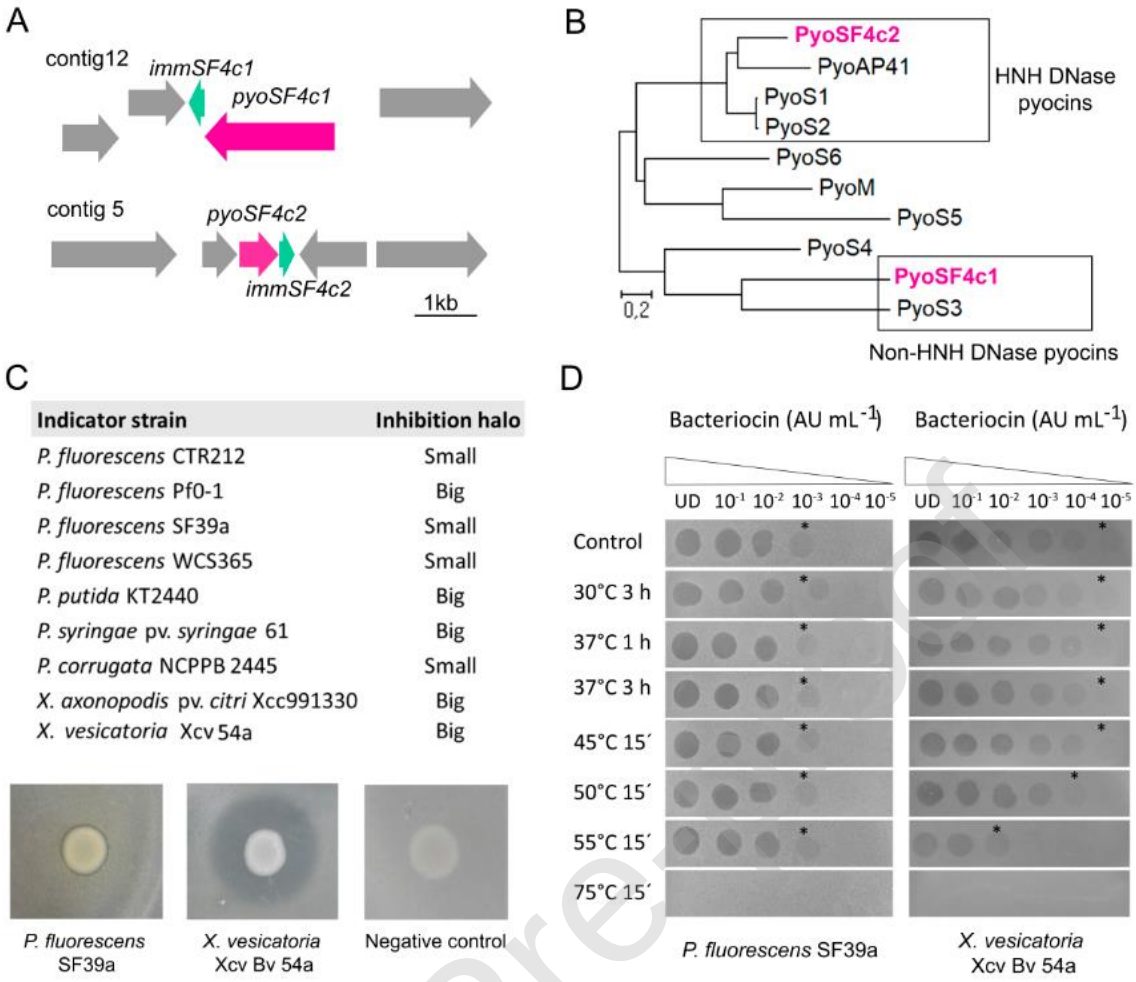


Fig. 2. Detection of tailocins in *P. fluorescens* SF4c.

Panel A. Schematic representation of the general organization of the tailocin cluster in *Pseudomonas fluorescens* SF4c.

Panel B. Bacteriocin proteins identified in samples obtained from cultures of strain SF4c (both induced with mitomycin C and uninduced) by nanoLC-MS/MS.

Panel C. Fold increase of bacteriocin proteins in mitomycin-C–induced samples compared to uninduced samples.

Panel D. Bacteriocin titer from mitomycin-C–induced and uninduced samples. In the figure, the inhibition of *X vesicatoria* is displayed for the concentrations in arbitrary units (AU) indicated above the series of photographs. UD: undiluted.

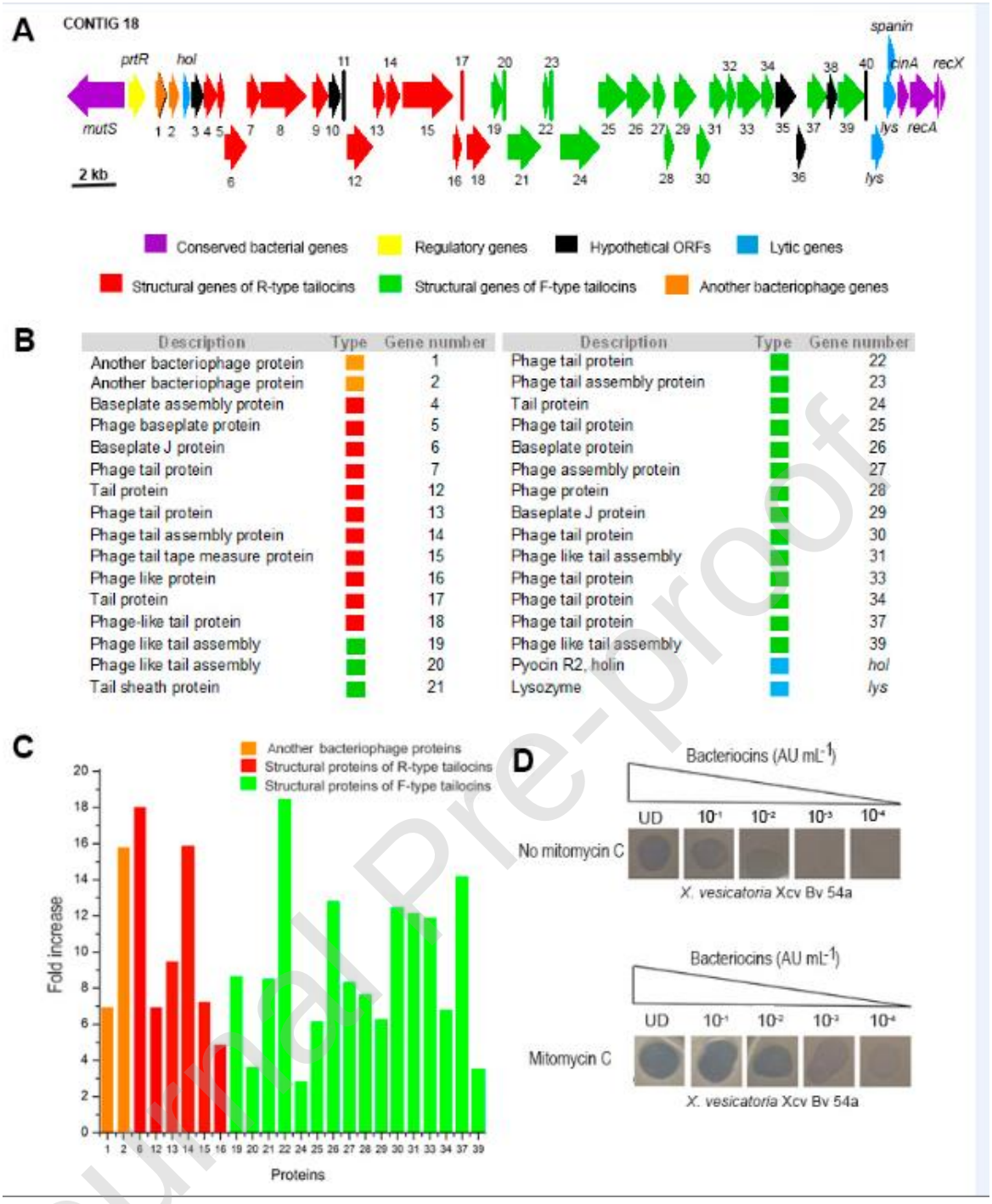


Fig. 3. Identification of the first gene of SF4c tailocin by RT-PCR.

Panel A. Schematic map of the chromosomal region where *orf* 1, *orf* 2, and *hol* are located. The black lines and opposing arrowheads below the map indicate the approximate locations of the oligonucleotide primers used for the RT-PCR and the resulting fragment sizes in regions a, b, and c.

Panel B. Electrophoresis of the RT-PCR products obtained with RNA from cultures of *Pseudomonas fluorescens* SF4c at an OD_{600} of 1.8 as a template and primers designed to amplify the cDNAs from the mRNAs corresponding to the *orf* 1–*orf* 2 intergenic region

(lanes 2 and 4; expected size, 496 bp), the *orf* 2–*hol* intergenic region (lanes 6 and 8; expected size, 357 bp), and *hol* (lanes 10 and 12; expected size, 236 bp). Lanes 4, 8, and 12 are controls without reverse transcriptase (with Taq polymerase) to ensure that amplification was not result from residual DNA. Lanes 3, 7, and 11 are negative controls with reverse transcriptase alone. Lanes 5, 9 and 13 are controls without reverse transcriptase, but with the Taq polymerase and DNA to insure the system's ability to amplify. Lanes 1 and 14 contain the molecular-weight markers. The length of the visible fragments is given in bp.

The symbols (-) and (+) indicate negative and positive controls, respectively.

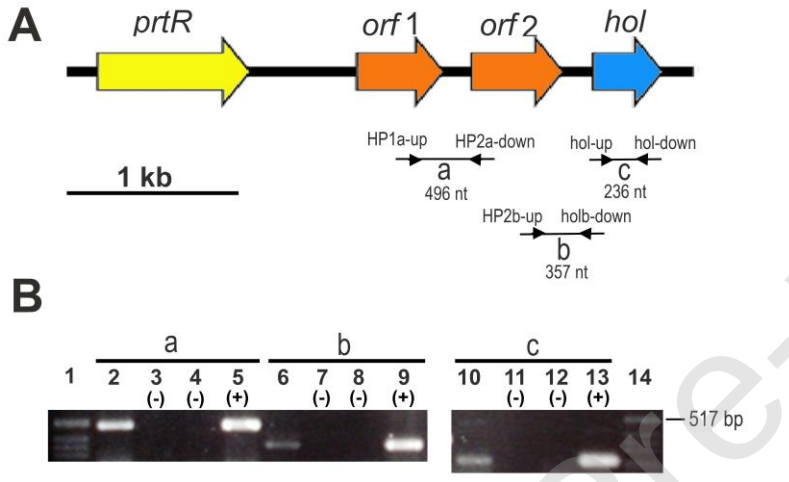


Fig. 4. Tailocin promoter of *P. fluorescens* SF4c.

Panel A. Nucleotide sequence of putative promoters P_{tailI} and P_{tailII} denoted by gray and green backgrounds, respectively, with the proposed –10 and –35 boxes underlined. The putative Shine-Dalgarno (SD) sequence and the start codon of *orf 1* are both labelled in red, while the primers to amplify P_{tailI} and P_{tailII} are marked in blue and pink, respectively.

Panel B. ß-Galatosidase activity from cultures of strain SF4c transformed with pMP220, pMP220::P_{tail}, pMP220::P_{tailI}, or pMP220::P_{tailII}, induced with mitomycin C (gray bars) or left uninduced (black bars). In the figure, the ß-galactosidase activity in Miller units per min per mg protein is plotted on the *ordinate* for the different cultures indicated on the *abscissa*. Strain SF4c, bearing the vector pMP220 empty was used as a control. Images of the LB plates supplemented only with X-gal (30 µg.mL^{-1}) or X-gal and mitomycin C (3 µg.mL^{-1}) are shown above the figure, with each plate corresponding to the treatment denoted by the bar directly below in the figure.

Three biological and technical replicates were performed for each treatment, and the experiment was conducted three times.

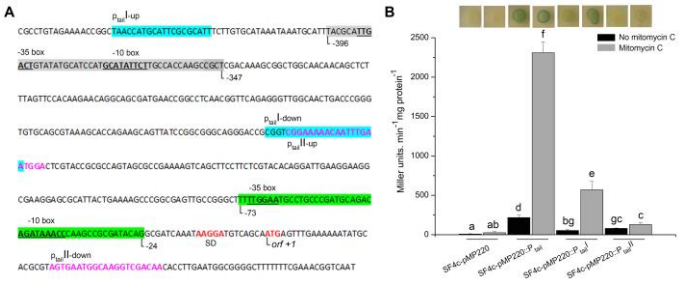


Fig. 5. Tailocin-promoter activity in mutant SF4c-prtR. In the figure, the expression of the green-fluorescent-protein (GFP) from mitomycin-C–induced cultures of strains SF4c, SF4c-prtR, and SF4c-prtR-c each carrying either the tailocin-promoter–bearing plasmid pPROBE::P_{tail} or the empty plasmid pPROBE-TT' (for the negative control), is measured at 490 nm and expressed as arbitrary units normalized at OD600 nm. Three biological and technical replicates were performed for each treatment, and the experiment was conducted three times. Different letters above the bars denote statistically significant differences in the extent of GFP expression and thus the level of promoter activity.

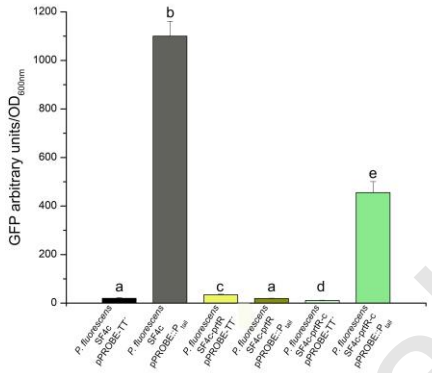


Table 1. *Bacterial strains and plasmids used in this study.*

Strains	**Origin or Relevant characteristics**	**Source or Reference**
P. fluorescens SF4c	Isolate from wheat rhizosphere (Argentina)	(Fischer et al., 2007)
P. fluorescens SF39a	Isolate from wheat rhizosphere (Argentina)	(Fischer et al., 2007)
P. fluorescens CTR212	Soil isolate (France)	(Latour et al., 1996)
P. fluorescens Pf0-1	Soil isolate (United States)	(Compeau et al., 1988)
P. putida KT2440	Plasmid-free derivative of natural isolate mt-2	(Franklin et al., 1981)
P. syringae pv. *syringae* 61	Isolate from wheat, pathogen (USA)	(Huang et al., 1988)
P. corrugata NCPPB 2445	Isolate from tomato, pathogen (UK)	(Scarlett et al., 1978)
X. axonopodis pv. *citri* Xcc991330	Isolate from *Citrus sinensis*, pathogen	INTA Bella Vista, Argentina
X. vesicatoria Xcv Bv5-4a	Isolate from pepper plants, pathogen	INTA Bella Vista, Argentina
P. fluorescens SF4c-prtR	Non-polar *prtR::Km* mutant of *P. fluorescens* SF4c, Km^R	This work
P. fluorescens SF4c-prtR-c	Non-polar *prtR::Km* mutant complemented with *prtR*, Km^R, Gm^R	This work
E. coli DH5α	endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1 Δ(lacZYA-argF)U169 φ80d lacZ Δ M15.	(Hanahan, 1983)
Plasmids		
pMP220	Promoter-probe vector containing a promoterless *LacZ* gene, Tc^R	(Spaink et al., 1987)

pMP220::P_{tail}	Putative promoters of the tailocins from *P. fluorescens* SF4c cloned into pMP220, Tc^R	This work
pMP220::P_{tailI}	The first putative promoter of the tailocins from *P. fluorescens* SF4c cloned into pMP220, Tc^R	This work
pMP220::P_{tailII}	The second putative promoter of the tailocins from *P. fluorescens* SF4c cloned into pMP220, Tc^R	This work
pUC18Not	Cloning vector, pUC18 derivative with polylinker flanked by *Not*I sites	(Herrero et al., 1990)
p34S-Km3	Plasmid carrying a Km^R cassette flanked by duplicated restriction sites	(Dennis and Zylstra, 1998)
pKNG101	Suicide vector for gene replacement; Sm^R; *sacB*	(Kaniga et al., 1991)
pPRTR2	907 bp *Bam*HI/*Kpn*I fragment of *prtR* cloned into pUC18Not	This work
pPRTR21	1330 bp *Hind*III/*Bam*HI fragment of *prtR* cloned into pPRT2	This work
pPRTR21Km	*Bam*HI fragment containing Km^R cassette from p34S-Km3 cloned into pPRTR21	This work
pKNGPRTR	*Not*I fragment of pPRTR21Km cloned into pKNG101, Sm^R	This work
pME3280a	Chromosomal integration vector, mini-Tn7 Gm^R , MCS	(Zuber et al., 2003)
pPrtR	Plasmid containing *prtR* and 300 bp upstream of this gen from *P. fluorescens* SF4c cloned into pME3280a, Gm^R	This work
pUX-BF13	Helper plasmid encoding Tn7 transposition functions, Ap^R	(Bao et al., 1991)
pPROBE-TT’	Promoter-probe vector based on stable GFP, Tc^R	(Miller et al., 2000)
pPROBE::P_{tail}	P_{tail} promoter from *P. fluorescens* SF4c	This work

	cloned into pPROBE-TT', Tc^R	

Ap^R, ampicillin resistant; Gm^R, gentamicin resistant; Km^R, kanamycin resistant; Sm^R streptomycin resistant; Tc^R, tetracycline resistant.

Table 2. *Antibacterial activity of culture of P. fluorescens SF4c and mutant SF4c-prtR mitomycin-C-induced* or *uninduced.*

	Tailocin titre (AU mL^{-1})			
	Uninduced		**Mitomycin-C-induced**	
	SF4c	**SF4c-prtR**	**SF4c**	**SF4c-prtR**
P. fluorescens CTR212	10^1	-	10^4	-
P. fluorescens SF39a	10^2	-	10^4	-
X. vesicatoria Xcv Bv5–4a	10^2	-	10^4	-

The symbol (-) indicates no growth inhibition of indicator strain.